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In situ reverse transcription–PCR in plant tissues

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▼*In situ* reverse transcription–polymerase chain reaction (RT–PCR) is a powerful technique which reveals the spatial location of low-abundance mRNA species by PCR amplification of the corresponding cDNA in paraffin-embedded tissues. This technique is widely used for the detection of RNA viruses and in studies of gene expression in fixed animal tissues and cells (Ref. 1, 2). Except for a description of a radioactive method in *Arabidopsis* (Ref. 3), there are no published reports on the application of this methodology to plant tissues. Because of our interest in establishing the spatial location of the mRNA product of an extremely poorly expressed regulatory gene, we have adapted the technique for plant tissues.

The *Pl* gene, a *myb*-like regulatory gene belonging to the *C1/Pl* family, controls the accumulation of anthocyanins in different maize (*Zea mays*L.) seed and plant tissues (Ref. 4). Dominant and recessive alleles at this locus are known which differ in their tissue specificity and response to light. Previous attempts to visualize the transcripts of the *Pl* gene by *in situ* hybridization were unsuccessful probably because of the low abundance of the corresponding mRNA.

We report here a procedure that allows determination of the spatial location of the *pl* allele transcripts within the seed. In presence of light, the *pl* allele controls pigmentation of different plant tissues, including the pericarp layer, and its expression was previously detected only by RT–PCR (Ref. 5). All the steps of the procedure are described in the protocol given below .

The results of the direct *in situ* RT–PCR experiment are shown (Fig. 1b, c). A cytoplasmic signal is clearly visible in all layers of the pericarp at the periphery of the cells over or around nuclei. The pericarp cells are highly vacuolated and, since the cytoplasm is confined to a thin peripheral layer, the signal tends to overlap the nucleus. A few stained nuclei are evident in the nucella. The presence of *pl* transcripts in the nucella cells may be due to the common origin of the two tissues since both the pericarp and the nucella are maternally derived. By contrast, the aleurone and the peripheral layers of the endosperm do not show any signal indicative of the strict tissue-specific expression of the *pl* allele. The dark irregular bodies, morphologically quite different from nuclei, visible in the endosperm on the left side of (Fig. 1b) may be light-refracting starch grains damaged by the procedure. In the positive control (Fig. 1d, e) DNase digestion and reverse transcription omitted), the great majority of nuclei of all tissues (pericarp, nucella, aleurone, endosperm) are darkly stained thus demonstrating optimal protease digestion, DNA amplification and detection. A negative control obtained by omitting the reverse transcription step is shown (Fig. 1g, f). In contrast to the positive control (Fig. 1d), the presence of unstained nuclei in the aleurone and sub-aleurone cells of the endosperm demonstrates the lack of non-specific amplification of nuclear DNA. The dark bodies observed in the test slide are also present in the negative controls.

The results reported demonstrate that it is possible to visualize low-abundance transcripts by *in situ* RT–PCR in plant tissues and that this approach provides a powerful tool for the analysis of gene expression.

Protocol

Tissue preparation and pretreatment

1. Immature homozygous *pl* kernels (19 days after pollination) were exposed to light for 48 h, fixed for 24 h in 4% paraformaldehyde (Merck) in phosphate-buffered saline (PBS, pH 11) (Ref. 6), dehydrated and embedded in Paraplast Plus (Sherwood Medical). Four sections 8-μm thick were placed on each organosilane-coated RNase-free glass slide.
2. Sections were deparaffinized with xylene for 20 min and rehydrated through an ethanol series. The slides were washed sequentially in distilled water 4 min,

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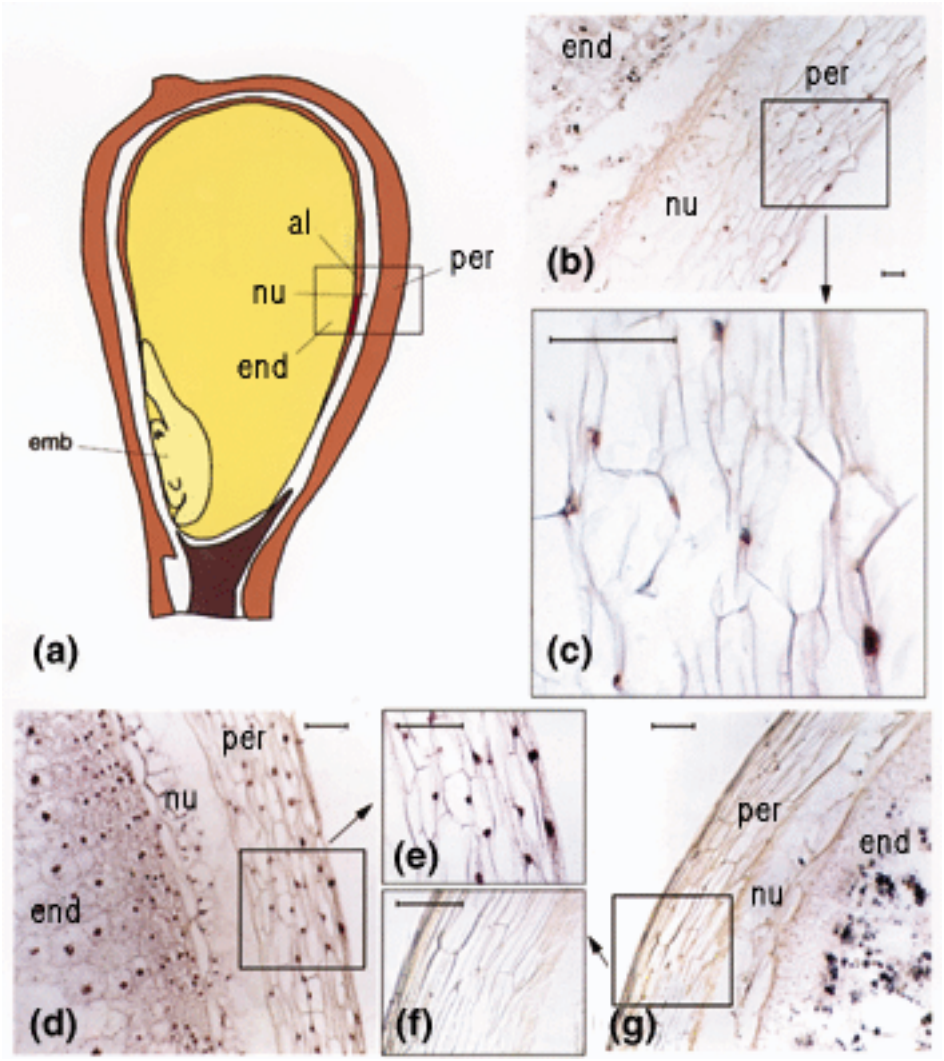

FIGURE 1. Localization of *pl* transcripts in the pericarp of a developing maize seed visualized by *in situ* RT—PCR. (a) Schematic drawing of the morphology of an immature maize seed (19 days after pollination). The rectangle denotes the region of the seed presented in the photographs which show the arrangement of the different tissues analyzed. (b) *In situ* RT—PCR revealing a specific signal in cells of the pericarp. (c) Enlargement of (b). (d) Positive control demonstrating stained nuclei in all cell types. (e) Enlargement of (d). (g) Negative control (DNase, RT omitted) showing that nuclear signals are lost in all cells. (f) Enlargement of (g). Bars = 50 μm. al, aleurone layer; emb, embryo; end, endosperm; nu, nucellar tissue; per, pericarp.

0.2 M HCl 20 min, 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH7) 30 min, distilled water 5 min, PBS 5 min.

3. Slides were post-fixed in 4% paraformaldehyde in PBS (pH 7.5) for 5 min at room temperature and washed for 5 min in PBS.

Permeabilization

It is important to optimise this step for each cell type or tissue sample because as well as facilitating the entry of reagents, permeabilization may also allow the exit of small amplification products. In our case, tissues were incubated in a humid chamber with 2 μg/ml proteinase K (Sigma) in 100 mM Tris HCl, 50 mM EDTA (pH 8) for 15 min at 37°C and then washed in PBS for 5 min and 2 mg/ml glycine (Merck) in PBS for 1 min before air drying.

DNase digestion

1. In order to render the native DNA non-amplifiable, it was exposed for an extended period to a high concentration of DNase. Tissues were incubated in a humid chamber with a RNase-free DNase solution (Boehringer Mannheim) (10 U/slide) at 37°C overnight.
2. DNase activity was stopped by immersion in 0.5 M EDTA for 10 min and the slides were then washed in 1 × SSC for 5 min and in distilled water for 2 min and air dried.

Table 1. Nucleotide sequences of oligonucleotides used as primers

Oligo code	Sequence (5′–3′)
PI6	TCGGACGACTGCAGCTCGGC
PI4	CCGATACTATTTCATACGCATAC

The DNase step can be omitted if the primers to be used for amplification are able to amplify only the cDNA and not the genomic copy.

Reverse transcriptase

1. Tissue sections were covered with 70 μl of Solution 1 [1.2 μM oligo(dT) and distilled water], covered with a parafilm coverslip and incubated in an OmniSlide Thermal Cycler (Hybaid) for 10 min at 70°C.
2. After removing the coverslip, 30 μl of Solution 2 [200 U/slide AMV Reverse Transcriptase (Boehringer Mannheim), 2 U/μl RNasin (Boehringer Mannheim), 1 mM each of dATP dCTP dGTP dTTP, 10 mM dithiothreitol (DTT) and RT Buffer (final concentration 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $mgCl_2$] were added and the sections covered with a new piece of parafilm.
3. The slides then were maintained at room temperature for 10 min, at 42°C for 45 min and at 70°C for 10 min. The slides were then washed 5 times in 2× SSC, 1× SSC, 0.5× SSC and twice in distilled water before air drying.

Polymerase chain reaction

1. Before the *in situ* PCR experiments, all parameters for the PCR reaction must be optimized by solution-phase PCR. A PCR solution containing 2.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP and dTTP, 100 μM digoxigenin dUTP (Boehringer Mannheim), 1 μM of primers and 1 U Taq polymerase (Promega) in a total volume of 50 μl was applied to each slide. The slides were then covered by silanized glass coverslips, sealed with rubber cement and placed in an OmniSlide Thermal Cycler (Hybaid). PCR was performed by denaturation at 94°C for 5 min, followed by 20 cycles of denaturation at 94°C for 1 min 30 s, annealing at 62°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 15 min was performed to complete the reaction. For the target amplification, 20 cycles were adequate to obtain deep staining.
2. After DNA amplification, slides were washed five times in 2× SSC, 1× SSC, 0.5× SSC and twice with distilled water to eliminate unbound nucleotides and air dried.

Immunodetection of PCR products

Slides were equilibrated in maleate buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 5 min, incubated for 30 min with 2% sheep serum (Sigma) and 0.3% Triton-X100 dissolved in the maleate buffer and exposed for 3 h at 24°C to 100 μl of anti-digoxigenin [Fab'] antibody (Boehringer Mannheim) bound to alkaline phosphatase diluted 1:500 in maleate–sheep serum–Triton-X100 buffer.

Slides were washed in maleate–sheep serum–Triton-X100 buffer for 15 min and then in magnesium buffer (100 mM Tris HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 min.

After thorough rinsing, the appropriate substrates [nitroblue tetrazolium salt (Boehringer Mannheim) and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim)] were enzymatically transformed into a dark-blue precipitate. The chromogenic reaction was halted by immersion in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) for 10 min followed by washing in distilled water for 2 min and air drying.

Controls

At least four different controls are needed to exclude false positive or negative results.

***Positive control*:** omission of the DNase and RT steps. An intense signal reveals optimal protease digestion, DNA amplification and detection. The signal will be completely eliminated with an overnight DNase digestion.

Negative control 1: omission of the RT reaction or RT with irrelevant primers will lead to unstained tissues. Staining of this control will reveal the presence of false positive results owing to amplification of endogenous DNA sequences.

Negative control 2: omission of primers in the PCR mixture reaction. Detection of a signal will reveal non-specific staining owing to the exonuclease activity of the DNA polymerase.

Negative control 3: omission of Taq polymerase. The presence of a signal will reveal non-specific binding of the primary antibody.

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Products Used

proteinase K: proteinase K from Sigma
proteinase K: proteinase K from Boehringer Mannheim
proteinase K: proteinase K from QIAGEN GmbH
Proteinase K: Proteinase K from PE Applied Biosystems
RNase-free DNase: RNase-free DNase from Boehringer Mannheim
RNase-free DNase: RNase-free DNase from Boehringer Mannheim
RNase: RNase from Sigma
RNase-free DNase solution: RNase-free DNase solution from Boehringer Mannheim
RNase-free DNase: RNase-free DNase from Promega Corporation
Thermal Cycler: Thermal Cycler from Techne (Cambridge) Ltd
thermal cycler: thermal cycler from MJ Research Inc
MMLV and AMV reverse transcriptase: MMLV and AMV reverse transcriptase from Promega Corporation
AMV reverse transcriptase: AMV reverse transcriptase from Boehringer Mannheim
Reverse Transcriptase: Reverse Transcriptase from Boehringer Mannheim
AMV reverse transcriptase: AMV reverse transcriptase from Promega Corporation
RNAsin: RNAsin from Promega Corporation
RNasin: RNasin from Boehringer Mannheim
Anti-digoxigenin antibody: Anti-digoxigenin antibody from Boehringer Mannheim
Taq DNA polymerase: Taq DNA polymerase from PE Applied Biosystems
Taq DNA polymerase: Taq DNA polymerase from Life Technologies (Gibco BRL)
Taq DNA polymerase: Taq DNA polymerase from Life Technologies (Gibco BRL)
Taq DNA polymerase: Taq DNA polymerase from Promega Corporation
Taq polymerase: Taq polymerase from Boehringer Mannheim
Taq polymerase: Taq polymerase from Pharmacia
Taq polymerase: Taq polymerase from Bioline
Taq polymerase: Taq polymerase from Advanced Biotechnologies
Taq polymerase: Taq polymerase from Boehringer Mannheim
Taq polymerase: Taq polymerase from Bioline
Thermal Cycler: Thermal Cycler from Techne (Cambridge) Ltd
thermal cycler: thermal cycler from MJ Research Inc
sheep serum: sheep serum from Sigma
Anti-digoxigenin antibody: Anti-digoxigenin antibody from Boehringer Mannheim
nitroblue tetrazolium salt: nitroblue tetrazolium salt from Boehringer Mannheim
5-bromo-4-chloro-3- indolylphosphate: 5-bromo-4-chloro-3- indolylphosphate from Boehringer Mannheim